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TITLE: Preclinical Development of TVAX: An Advanced Multiantigen Vaccine for Therapy and Prevention of Malignant Mesothelioma

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14. ABSTRACT We proposed to evaluate the efficacy of a multi-antigen vaccine for the treatment of malignant mesothelioma (MM) in mice. The first version of this vaccine, called mTvax, included epitopes to activate antigen-specific T cells against survivin, metastasin, midkine, Wilm's Tumor 1, brachyury, Fibroblast Activation Protein and VEGFR2. In mTvax we also included the immunostimulatory molecules CD80, CD54, and CD48 with the purpose of improving T cells responses. The mTvax antigen, comprising of the epitopes for T cell activation and the three immunostimulatory molecules, has been inserted into the DNA of different vectors (p-mTvax, MVA-mTvax and FP-mTvax). When experiments were performed to evaluate the efficacy of mTvax vaccines, only FP-mTvax induced a statistically significant delay in tumor progression. Moreover, when we combined FP-mTvax with OX40 agonist antibodies (OX86), we did not observe any reduction in tumor growth. To investigate our hypothesis that CD80, CD54, and CD48 reduce the efficacy of our vaccines, we produced mTvax 2.0 that expresses the same cancer antigen of mTvax but lacks the three immune stimulatory molecules. Experiments performed using mTvax 2.0 in mice carrying MM tumors showed vaccine-induced specific T cell responses and delay in tumor growth using both MVA and FP vectors.					
15. SUBJECT TERMS Malignant Mesothelioma, anti-cancer vaccines, T cell-epitope, multi-epitope vaccines, T regulatory cells, tregitopes.					
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INTRODUCTION:

Malignant Mesothelioma (MM) is an aggressive form of cancer that typically originates in the pleural but can also occur in the peritoneum, pericardium and around the testes. Asbestos exposure is the only established risk factor for MM (1).

Even if MM is a rare cancer, a significant number of MM deaths occur among U.S. navy sailors and military personnel that were heavily exposed to asbestos because they worked in shipyards or in their proximity (2). Since there are no effective treatments for MM and the median survival is less than 1 year from diagnosis, there is an urgent medical need to develop novel approaches to treat this understudied disease.

Our purpose is to use advanced anti-cancer vaccines to generate effective immune responses against MM cells. In clinical trials, conventional anti-cancer vaccines have shown to be safe and to induce tumor-specific T cell responses, although increase in survival has been achieved in a limited number of patients. Most of the vaccines utilized in clinical trials, as well in pre-clinical studies usually target a single cancer antigen. Our data obtained with survivin vaccine FP-surv suggest that vaccination with this single antigen was effective against survivin-positive cells (3). However, eradication of survivin-positive cells was followed by an expansion of cancer cell clones with reduced or undetectable survivin expression. These clones elude survivin-specific cytotoxic T cells and maintain the ability to grow uncontrolled, and express other proteins such as metastasin, midkine, wilms tumor-1 (WT-1) and brachyury. Although vaccines expressing these tumor antigens have been evaluated with promising results, all of them presented the same pattern of limitations observed for other single-antigen vaccines. In this project we generate more broad immune responses against several antigens with our next-generation vaccine called mTvax. Immunostimulatory epitopes of survivin, metastasin, midkine, WT-1 and brachyury have been incorporated in mTvax together with epitopes from Fibroblast Activation Protein (FAP) and vascular endothelial growth factor receptor 2 (VEGFR2). The last two antigens were added because cells that constitute the tumor stroma produce high amounts of FAP and the endothelial cells that form the tumor vasculature show high levels of VEGFR2. Together, FAP- and VEGFR2-positive tissues provide mechanical and nutritional support to the tumor and have been demonstrated to be promising targets for immunotherapy.

The scope of this research is to evaluate the therapeutic efficacy of mTvax and its activity on cancer-specific T cells in mouse MM models. We also proposed to develop a clinical version of mTvax (Tvax) and evaluate its efficacy in activating human T cells from healthy donors.

KEYWORDS:

Malignant Mesothelioma, anti-cancer vaccines, T cell-epitope, multi-epitope vaccines, T regulatory cells, tregitopes.

ACCOMPLISHMENTS:**What were the major goals of the project?**

Major Tasks for Year 1 as stated in the SOW:

- Major Task 1. Obtain regulatory approval for the use of animal.

Timeline: 1-6 months Milestone Achieved

- Major Task 2. Identify the most efficacious vaccine comprised of mTvax and OX86 antibodies designed to induce mesothelioma (MM)-specific T cells in BALB/c mice.

Previous Timeline: 6-9 months, modified to 6-15 months after change on SOW

- Major Task 3. Evaluate the efficacy of mTvax vaccines in subcutaneous tumor model of MM progression.

Previous Timeline: 9-12 months, modified to 9-15 months after change on SOW

What was accomplished under these goals?

As major activities during this reporting period, we evaluated the efficacy of mTvax vaccines in activating antigen-specific responses and delay cancer progression in mouse MM models. The first version of mTvax, included epitopes to activate antigen-specific T cells against survivin (BIRC5), metastasin (gene S100A4), midkine (gene Mdk), Wilm's Tumor 1 (gene WT1), brachyury (gene T), Fibroblast Activation Protein (gene FAP) and VEGFR2 (gene Kdr). In mTvax, we also included the immunostimulatory molecules CD80, CD54, and CD48 with the purpose of improving T cells responses. The mTvax antigen, comprising of the epitopes for T cell activation and the three immunostimulatory molecules, has been inserted into the DNA of three different vectors: a plasmid DNA vector (p-mTvax) and two viral vectors (MVA-mTvax and FP-mTvax).

Our objective was to compare anti-cancer activities of the different mTvax vectors alone or with OX40-agonist antibodies (OX86). We also tested the combination of the different mTvax vectors in a heterologous prime-boost setting. This immunization strategy has been demonstrated to extend the activation of cancer-specific T cells and can be applied to achieve durable anti-cancer responses.

We performed experiments in which BALB/c mice carrying MM subcutaneous tumors were vaccinated with different immunization schedules. In these mice, we evaluated differences in tumor growth and survival.

The results of these experiments indicated:

- a) Activities of viral vaccine vectors as MVA and FP were consistent in repeated experiments. In the other hand, plasmid DNA vaccines showed variable results with significant anti-cancer responses achieved only in few mice for each experiment. To investigate this issue, we analyzed levels of antigen expression after subcutaneous (s.c) injection of p-mTvax in BALB/c mice. As a reporter antigen, we included luciferase in p-mTvax and evaluated its expression using an IVIS imaging system after intraperitoneal (i.p.) injection of luciferin. As showed in Figure 1, some of the mice injected with p-mTvax did not present any luciferase activity. We concluded that s.c. injection of plasmid

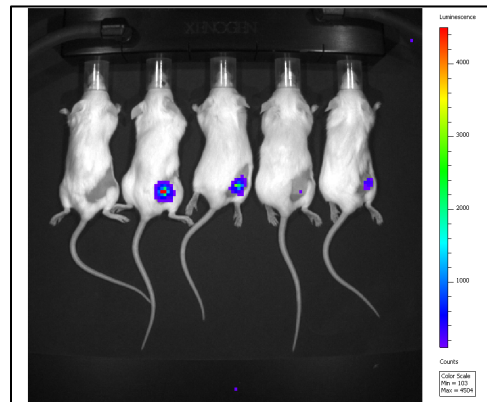


Figure 1. Luciferase activity in mice injected with p-mTvax. Mice were injected s.c. with 5 μ g of p-mTvax carrying the luciferase gene as reporter antigen. Luciferase expression has been evaluated with an IVIS imaging system after injection of 100 μ l of luciferin.

DNA is not a reliable way to vaccinate mice. Different inoculation methods could be investigated for plasmid DNA vaccines, as Gene gun or *in vivo* electroporation. However, to establish these *in vivo* transfection methods we should acquire instruments that would have a substantial impact on the budget. Viral vector vaccines (MVA and FP) instead demonstrated consisted results and can be produced in our laboratories without additional expenses. For these reasons, we decided to use MVA and FP vectors as leading vaccine platforms for this project.

b) Vaccination with FP-mTvac as treatment with OX86 antibodies delay tumor growth and extend survival of mice carrying MM tumors (Fig.2).

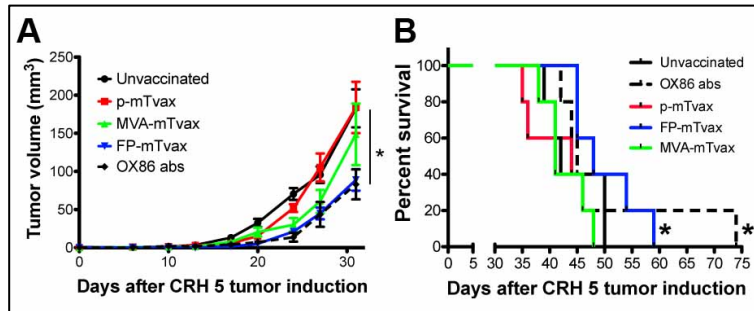


Figure 2. FP-mTvac and OX86 abs delay tumor growth and improve survival of mice carrying MM tumors. BALB/c mice were injected with 100,000 CRH5 MM cells and treated at day 7 and 14 with mTvac vaccines or OX86 abs. Tumor growth was measured with a caliper (A) and survival evaluated with mice euthanized when tumors reached 200mm³. **P*≤0.05

c) Vaccination with FP-mTvac in combination with OX86 antibodies does not reduce tumor growth (Figure 3).

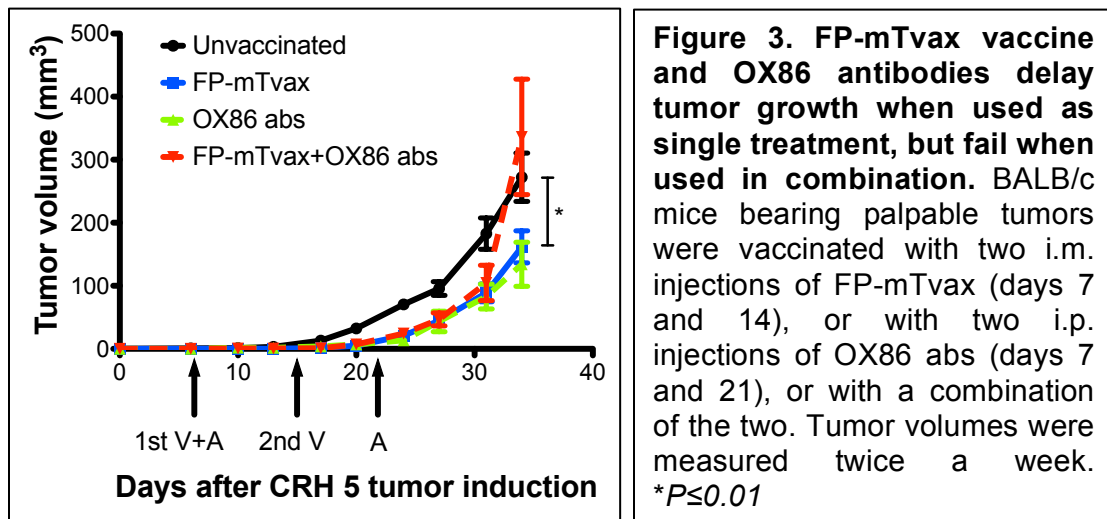


Figure 3. FP-mTvac vaccine and OX86 antibodies delay tumor growth when used as single treatment, but fail when used in combination. BALB/c mice bearing palpable tumors were vaccinated with two i.m. injections of FP-mTvac (days 7 and 14), or with two i.p. injections of OX86 abs (days 7 and 21), or with a combination of the two. Tumor volumes were measured twice a week. **P*≤0.01

We hypothesized that the immunostimulatory molecules CD80, CD54, and CD48 included in the mTvac antigen, may contribute to the loss of efficacy showed by FP-mTvac after OX40 engagement with OX86 antibodies. It has been demonstrated that self-full-length protein as CD80, CD54 and CD48 may contain T cell epitopes, called tregitopes, that specifically activate regulatory T cells (Tregs) (4). The activation of Tregs by tregitopes may increase during OX40 engagement and explain the lack of FP-mTvac efficacy observed in Figure 3. The reduced activity of FP-mTvac after OX40 engagement can also be caused by autoimmune responses targeting immune cells expressing CD80, CD54, and CD48.

To investigate our hypothesis that CD80, CD54, and CD48 reduce the efficacy of our vaccines, we produced **mTvac 2.0** that expresses the same cancer antigens of mTvac but lacks the three immune stimulatory molecules. The mTvac 2.0

antigen has been inserted into MVA and FP vectors (MVA-mTvax 2.0 and FP-mTvax 2.0) and utilized to vaccinate mice carrying subcutaneous MM tumors. In these mice, we evaluated tumor dimension, survival and T cell immune responses.

The results of the experiments performed with mTvax 2.0 indicated:

- a) Both MVA-mTvax 2.0 and FP-mTvax 2.0 delayed tumor growth and improved survival of mice carrying MM tumors. The efficacy of both mTvax 2.0 vaccines was significantly improved by treatment with OX86 antibodies (Figure 4).

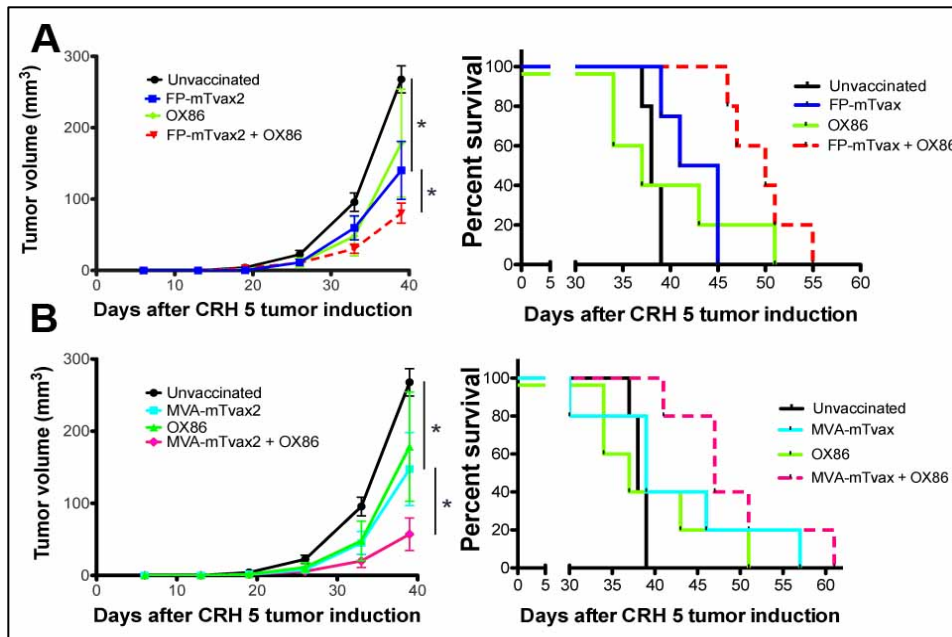


Figure 4. mTvax 2.0 vaccines delay tumor growth and improve survival when used as single treatment or in combination with OX86. BALB/c mice bearing palpable tumors were vaccinated with two i.m. injections of mTvax2 vaccines (MVA or FP in days 7 and 14), or with two i.p. injection of OX86 abs (days 7 and 21), or with a combination of the two. Tumor volumes were measured with a caliper and survival evaluated with mice euthanized when tumors reached 200mm³. **P*≤0.01

- b) mTvax 2.0 induces epitope-specific T cell immune responses in BALB/c mice. This was evaluated by flow cytometry analysis of T cells from vaccinated mice stimulated with each the 42 epitopes included in mTvax 2.0. In both CD4⁺ and CD8⁺ T cells, we measured production of IFN γ as an index of immune activation. To investigate the activation of T regulatory cells, we instead measured production of IL-10 and TGF β . The results of this analysis revealed that 13 peptides of those produced by mTvax 2.0 stimulate the production of IFN γ in a significant number of CD4⁺ and CD8⁺ T cells. Those peptides were distributed among all the

seven target antigens included in mTvax 2.0. None of the peptides representing the epitopes in mTvax 2.0 induced the production of IL-10 or TGF β in any of our experiments. A representative image of the flow cytometry analysis of CD4⁺ T cells after stimulation with the Brachyury peptides is shown in Figure 5. An example of the data obtained for the mTvax 2.0 target-antigen Survivin, is instead shown in Figure 6. The results of the experiments using peptides from the other mTvax 2.0 target-antigens were not shown.

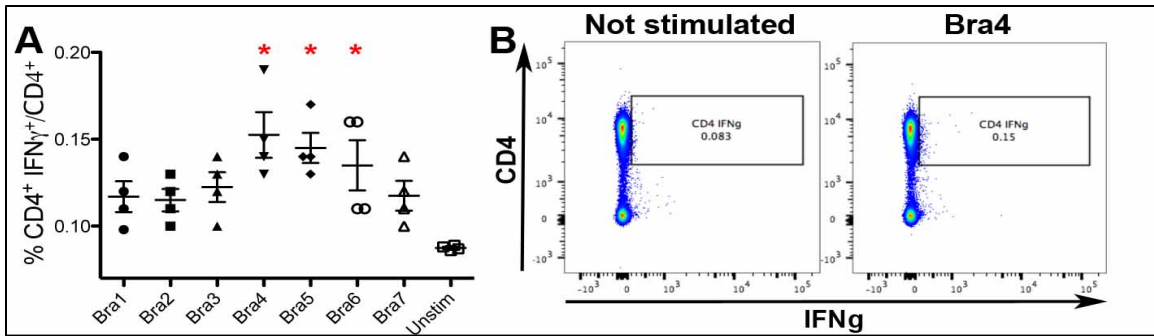


Figure 6. Bracyury peptides induce epitope-specific CD4⁺ T cell immune responses in BALB/c mice vaccinated with MVA-mTvax 2.0. Groups of 5 mice were vaccinated with MVA-mTvax 2.0 (10e6 pfu) on day 1 and 7. Lymph nodes were harvested on day 17 and immune cells stimulated with 7 different Bracyury peptides representing the same Brachyury epitopes included in mTvax 2.0. After 6 hours of incubation with Brefeldin A, cells were stained for the indicated antibodies and analyzed at the flow cytometer. A) Percentages of CD4⁺ IFN γ ⁺ cells after stimulation with the different Brachyury peptides. Unstimulated cells were used as control. * $P \leq 0.01$ B) Representative image from the flow cytometry analysis.

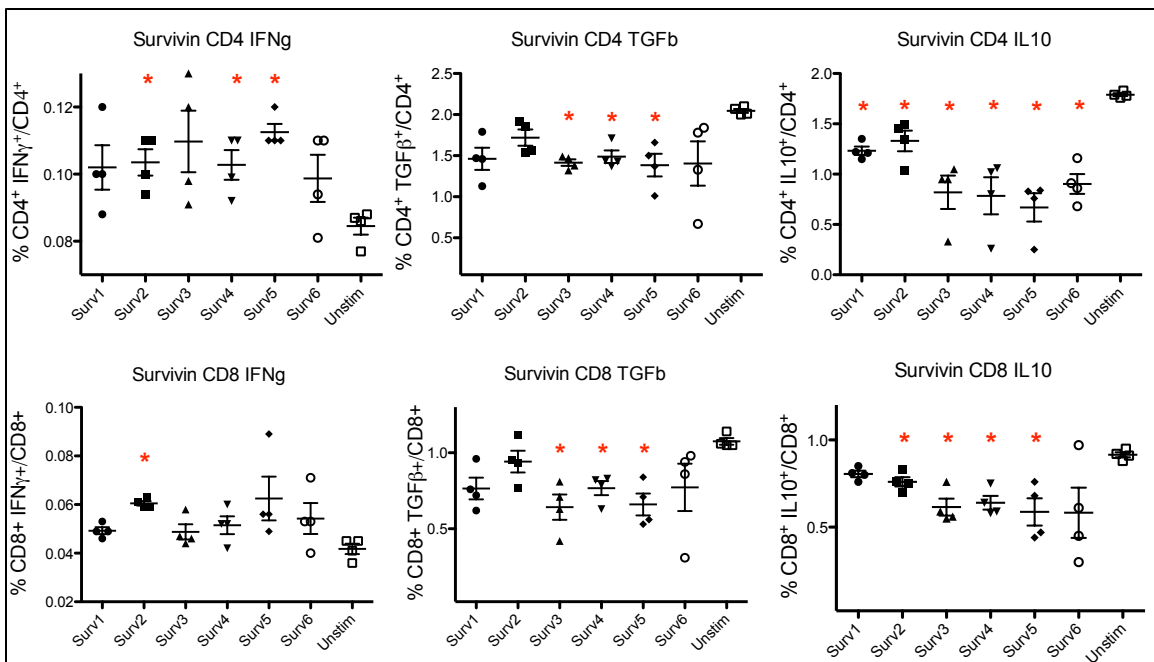


Figure 7. Survivin peptides induce epitope-specific T cell immune responses in mice vaccinated with MVA-mTvax 2.0 without activating T regulatory cells. Groups of BALB/c mice were vaccinated with MVA-mTvax 2.0 and lymph nodes harvested ten days later. Immune cells were isolated and stimulated with 6 different Survivin peptides representing the same Survivin epitopes included in mTvax 2.0. Control cells were not stimulated (Unstim) After 6 hours of incubation with Brefeldin A, cells were stained for the indicated antibodies and analyzed at the flow cytometer. Statistical differences between each Survivin peptide and not stimulated controls were evaluated with ANOVA (* $P \leq 0.01$).

In conclusion, in our experiments we compared different immunization protocols using two mTvax vaccines: mTvax and mTvax 2.0. mTvax demonstrated limited efficacy in terms of tumor reduction and improved survival. FP-mTvax was the only vector that induced statistically significant results, but its effects vanished when combined with OX86 antibodies. In the other hand, mTvax 2.0 showed significant anti-cancer responses with both MVA and FP vectors, and their efficacy was augmented by OX86. mTvax 2.0 also induced epitope-specific T cell responses for all the target antigens, without generating IL-10⁺ or TGFβ⁺ T regulatory cells.

Even if these results obtained with mTvax 2.0 were promising, we expected stronger anti-cancer properties from a vaccine that target multiple antigens. To understand if the reduced efficacy of mTvax 2.0 is due to a limited expression of its target antigens in the tumor tissue, we measured mRNA expression of MM tumors and normal tissues using Clariom S gene array. We evaluated the expression of 40,000 mRNA and together with the Bioinformatics core at our institution we calculated tumor vs. normal fold changes in expression for each mRNA. As shown in Table 1, only three of the seven antigens included in mTvax 2.0 were expressed higher in CRH5 tumors than in normal tissues. Moreover, when we sorted all the genes based on their tumor vs. normal fold change mRNA expression, we found that in our cancer models there are several other antigens with higher tumor vs. normal fold change than those we included in mTvax 2.0.

Table 1. mRNA expression in CRH5 tumors and normal tissues measured with Clariom S microarray. The first row indicates the position for each gene in a list sorted from the highest tumors vs. normal fold change (Position 1) to the lowest (Position 39,998).				
Position	CRH5 vs normal. Fold Change	Normal	CRH5	Gene Symbol
28	25.4712	6.4871	11.1579	S100a4
337	6.5087	3.298	6.0004	Fap
494	4.9774	7.2925	9.6079	Birc5
21682	-1.2182	4.5352	4.2505	T
25740	-1.7431	6.6504	5.8488	Wt1
26857	-2.3683	8.0068	6.7629	Mdk
27946	-4.6569	11.4069	9.1876	Kdr

With the results obtained from the Clariom S assay, we are now designing mTvax 3.0 that will include an optimal selection of antigens with the highest

tumor vs. normal fold change. The epitopes from each target antigen will be identified using EpiVax immune-informatics tools, including the last application “Janus Matrix” that has been recently added for mouse vaccine development and improve selection of highly immunogenic T cell epitopes (5). The epitopes for both CD4⁺ and CD8⁺ activation will be included in one single transgene using the same structure of mTvax 2.0. Both MVA-mTvax 3.0 and FP-mTvax 3.0 will be produced and their anti-cancer properties compared with mTvax 2.0 vaccines.

During this reporting period (May 2017), we submitted a change in SOW to add experiments with mTvax 2.0 and mTvax 3.0. The change in SOW has been approved and the timelines for Major Tasks 2 and 3 postponed to 6-15 months. Major Tasks 4 and 5 in the next reporting period will be conducted using the mTvax vaccine that shows higher anti-cancer properties and induces stronger T cell immune responses.

What opportunities for training and professional development has the project provided?

A career development plan for myself, the PI of this project, was included in the proposal for this DoD grant. During this reporting period, I had weekly one-on-one meetings with my mentor, Dr. Hoffmann, to discuss analyses of data and the ongoing planning of this project. I also met once a month with my secondary mentor Dr. Carbone to analyze the histological samples obtained from the mouse studies and discuss the future direction of my researches.

I participated in the weekly seminars hosted by the Cancer Center and the Medical School at the University of Hawaii. I also attended some of the webinars organized by EpiVax and the Institute of Immunology and Informatics at the University of Rhode Island.

To improve my experience in teaching, I presented lectures for the courses in “Cell and Molecular Biology” (CMB 622) and “Infection and Immunity” (TRMD 610) at the University of Hawaii.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

For the next reporting period, I plan to perform experiment to assess which vaccine between mTvax 2.0 and mTvax 3.0 induce stronger anti-cancer immune responses. Once the best vaccine is identified, I will proceed and perform the

experiments included in the Major Tasks 4 and 5 of the SOW. Our major goal is to prepare and submit a manuscript before the ending of the next reporting period.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The main goal of this translation proposal is to test innovative vaccines in animal models of MM progression to identify new therapeutic and preventive interventions. In the therapeutic setting, Tvx vaccines may be used to induce tumor regression in patients with MM at the early stages of disease. In the case of most late-state tumors, chemotherapy or surgery can be used to reduce tumor burden. In these patients, immunotherapy with Tvx vaccines can be used post-treatment to stimulate the immune system in killing remaining cancer cells and increase post-cancer survival. Our studies would also provide the framework for developing vaccines to prevent cancer development in people exposed to asbestos and/or genetically predisposed who are at high risk for developing MM in the future. Investigators at the University of Hawaii Cancer Center have recently discovered germline Bap1 mutations in families with high incidence of MM. In addition, two biomarkers, Soluble Mesothelin Related Peptides (SMRP) and Osteopontin (OPN) have garnered interest in recent years as a means of detecting recurrence of MM. This progress has enabled the use of PCR analysis to detect Bap1 mutations and the use of ELISA to evaluate SMRP and OPN protein levels as a means of identifying patients at high risk of developing MM for early intervention (6).

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

In May 2017 we submitted a change in SOW that was approved by the awarding agency Grants Officer. In the SOW we included experiments to compare efficacy of mTvx 2.0 and 3.0. This change was due to the limited anti-cancer efficacy demonstrated by the first version of mTvx vaccine in our experiments. The results of those experiments are described in the "ACCOMPLISHMENTS" section of this document.

Actual or anticipated problems or delays and actions or plans to resolve them

In the change of SOW submitted during this reporting period, we extended the timeframe for Major Tasks 2 and 3 of three months to perform the experiments with mTvx 2.0 and mTvx 3.0. The timeline for Major Tasks 4 and 5 were therefore reduced from 12-18 months to 15-18 months. We are confident that once the best vaccine between mTvx 2.0 and mTvx 3.0 will be identified, experiments to evaluate vaccine efficacies in late-stage tumors or intraperitoneal tumors can be successfully conducted in three months.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations

- **Journal publications.**
Nothing to Report
- **Books or other non-periodical, one-time publications.**
Nothing to Report
- **Other publications, conference papers, and presentations.**

Some of the data from this project were presented during the “Cancer Biology Winter Retreat” at the Cancer Center of the University of Hawaii the 17th of December 2016.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

During this reporting period, we produced MVA and FP vectors expressing the mTVAX 2.0 antigen. These vaccines will be available for other research projects performed in my lab or Dr. Hoffmann lab. In case of collaboration, this vaccine can be also shared with other laboratories at the Medical School and/or Cancer Center of the University of Hawaii.

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name: Pietro Bertino

Project Role: PI

Research Identifier (ERA Commons ID): pbertino

Nearest person month worked: 9

Contribution to the project: Dr. Bertino had overall responsibility for all aspects of the project. He conducted experiments and analyzed the data. He also was in charge of preparing regulatory approval requests and progress reports.

Funding Support: This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS: Not applicable

APPENDICES:

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